CHARACTERISATION OF RECOMBINANT IGG BINDING PROTEIN, FCγRIIA FOR BIOSENSOR DEVELOPMENT

GAN SHIN YI

UNIVERSITI SAINS MALAYSIA

2025

CHARACTERISATION OF RECOMBINANT IGG BINDING PROTEIN, FCγRIIA FOR BIOSENSOR DEVELOPMENT

by

GAN SHIN YI

Thesis submitted in fulfilment of the requirements
for the degree of
Doctor of Philosophy

September 2025

ACKNOWLEDGEMENT

I am greatly grateful for all the support and encouragement I have received throughout this challenging and long journey of my PhD research. Firstly, I would like to extend my appreciation to the Fundamental Research Grant Scheme (FRGS/1/2023/SKK06/USM/02/6) and the Higher Institution Centre of Excellence (HICoE) Grant (311/CIPPM/44001005) from the Malaysian Ministry of Education Higher Institution for their generous financial support. I am especially grateful to my supervisor, Assoc. Prof. Dr. Lai Ngit Shin, my co-supervisor, Assoc. Prof. Dr. Tye Gee Jun, and Dr. Chew Ai Lan, for their invaluable mentorship and support throughout this journey. I would also like to acknowledge Dr. Khairul Mohd Fadzli Mustaffa and Assoc. Prof. Dr. Aziah Ismail from INFORMM Kelantan for their kind support and guidance. A sincere appreciation goes to Dr. Tech. Peter Greimel for his mentorship and encouragement during my short-term IPA in RIKEN. His generous sharing of knowledge has inspired me to stay passionate and resilient in pursuit of my goals.

To my beloved parents and siblings, thank you for your unconditional love, patience, and belief in me. My heartfelt thanks also go to Henry Lim Zhen Xiang for his unwavering support, emotionally and physically. Thank you for being by my side, offering your strength and companionship. I would also like to express my appreciation to Ms. Jacqueline Mark Kar Kei, Ms. Chin Ming Hui, Mr. Chin Ding Sheng, Mr. Wong Zhenpei, Ms. Deeza Syafiqah Mohd Sidek, Dr. Ahmad Najib Mohamad, and Dr. Kasturi Selvam for their friendship and support throughout the research process. Lastly, I wish to thank all the INFORMMers, laboratory staffs, and administrative personnel who contributed their time and assistance over the past three years. Thank you all, sincerely, for being part of this journey.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF SYMBOLS

≈	Almost equal to
°C	Celcius
+	Plus
-	Minus
=	Equal to
>	More than
<	Less than
±	More or less
≤	Less than and equal to
3’	End of DNA at carbon-3 position in its sugar-ring structure
5’	End of DNA at carbon-5 position in its sugar-ring structure
OD_{405}	Absorbance observed at wavelength 405 nm
bp	Base pair
cm	Centimeter
g	Gram
h	Hour
kb	Kilo base pair
kDa	Kilo Dalton
L	Liter
M	Molar
mg	Milligram
mL	Milliliter
mm	Millimeter
mM	Millimolar
n	Sample size

ng	Nanogram
p	p-value
™	Trademark
V	Volt
A	Ampere
Ω	Ohm
∝	Proportionality
×	Multiply
x g	Times gravity
α	Alpha
β	Beta
γ	Gamma
ε	Epsilon
Δ	Delta
σ	Standard deviation
S	Slope
µg	Microgram
µL	Microliter
µm	Micrometer
µA	Microampere

LIST OF ABBREVIATIONS

3D	Three-dimensional
ABTS	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
AFM	Atomic force microscope
ADCC	Antibody-dependent cellular cytotoxicity
Ab	Antibody
Ag	Antigen
APC	Antigen-presenting cell
APS	Ammonium Persulfate
Asp	Aspartic acid
β2m	Beta-2-microglobulin
BGH	Bovine Growth Hormone
BSA	Bovine Serum Albumin
cDNA	Complementary DNA
CHO	Chinese hamster ovary
CMV	Human Cytomegalovirus
Cys	Cysteamine
CV	Cyclic voltammetry
DDI	DNA-directed immobilisation
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DPV	Differential pulse voltammetry
E. coli	*Escherichia coli*
EDC/NHS	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide/ N-hydroxysuccinimide
EDTA	Ethylenediaminetetraacetic acid
EIS	Electrochemical impedance spectroscopy
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal Bovine Serum
Fc	Fragment crystallizable region
FcγR	Fc gamma receptor
FcRn	Neonatal Fc receptor
Glu	Glutamic acid

Gly	Glycine
HlyE	Hemolysin E
His	Histidine
HRP	Horseradish peroxide
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IMAC	Immobilised metal affinity chromatography
IV	Intermediary vector
LB	Luria-Bertani
Leu	Leucine
MHC	Major histocompatibility complex
n.s.	Not significant
$Na_2H_2PO_4$	Sodium dihydrogen phosphate
NaOH	Sodium hydroxide
NaCl	Sodium Chloride
NC	Nitrocellulose membrane
NCBI	National Center for Biotechnology Information
Ni-NTA	Nickel- nitrilotriacetic acid
NK	Natural killer cell
OD	Optical density
P13K	Phosphoinositide 3-kinase
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Tween-20 detergent
PCR	Polymerase chain reaction
pH	Potential of hydrogen
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Standard error of mean
SH	Sulfhydryl group
SPR	Surface plasmon resonance
SWV	Square wave voltammetry
TBE	Tris borate EDTA buffer

TEMED	Tetramethylethylenediamine
T_m	Melting temperature
Trp	Tryptophan
Tris-HCl	Trisaminomethane hydrochloride
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
UV-Vis	Ultraviolet–visible
V:V	Volume to volume

LIST OF APPENDICES

PENCIRIAN PROTEIN REKOMBINAN PENGIKAT IGG, FCγRIIA UNTUK PEMBANGUNAN BIOSENSOR

ABSTRAK

Antibodi memainkan peranan penting dalam aplikasi diagnostik disebabkan oleh kepekaan khusus dan afinitinya yang tinggi terhadap antigen sasaran. Namun begitu, dalam pembangunan biosensor, khususnya untuk diagnostik titik penjagaan (point-of-care, POC), kaedah pengimobilisasian antibodi pada permukaan sensor merupakan halangan kritikal yang mempengaruhi sensitiviti, keboleh-ulangan, dan prestasi keseluruhan ujian. Teknik tradisional seperti penjerapan pasif atau penggandingan kovalen secara rawak sering menghasilkan antibodi yang tidak stabil dan tidak terorientasi dengan baik. Kesan daripada kelemahan ini termasuklah hingar latar yang tinggi, aktiviti pengikatan yang rendah, keboleh-ulangan yang lemah, serta had pengesanan yang masih tinggi iaitu dalam julat mikrogram hingga nanogram per mililiter. Kekangan ini menghadkan kebolehpercayaan serta aplikasi klinikal biosensor elektrokimia. Bagi mengatasi kekangan ini, kajian ini menilai penggunaan reseptor Fc IgG manusia rekombinan, iaitu FcγRIA, FcγRIIA dan reseptor neonatal Fc (FcRn), sebagai ligan penangkap antibodi secara spesifik terhadap tapak Fc untuk aplikasi biosensor elektrokimia. Reseptor ini diekspreskan dalam sistem ekspresi mamalia bagi memastikan lipatan protein dan pengubahsuaian pasca-translasi yang betul. Walau bagaimanapun, penghasilan protein rekombinan adalah terhad akibat pengekalan intraselular, berkemungkinan disebabkan oleh peptida isyarat endogen yang tidak diubah suai serta domain transmembran yang bersifat hidrofobik. Selepas penulenan, reseptor-reseptor ini telah diimobilisasikan pada permukaan elektrod emas melalui ikatan kimia tiol-emas untuk memfasilitasi pengikatan antibodi secara berarah

melalui rantau Fc. Elektrod ini seterusnya digunakan dalam platform biosensor elektrokimia bagi pengesanan dua antigen model, iaitu interferon-gamma (IFN-γ) dan hemolisin E (HlyE). Prestasi sensor ini dinilai menggunakan voltametri gelombang persegi (SWV) dan voltametri kitaran (CV), manakala parameter analitikal seperti had pengesanan (LOD), had pengkuantitian (LOQ), dan pekali variasi (COV) telah dikira untuk menilai sensitiviti dan keboleh-ulangan. Antara ketiga-tiga reseptor, FcγRIIA menunjukkan prestasi terbaik dengan isyarat tidak spesifik yang minimum. Platform ini mencapai LOD 2.23 ng/mL bagi IFN-γ dalam larutan penimbal dan 1.71 ng/mL bagi HlyE dalam matriks serum, dengan nilai COV kurang daripada 15%. Penyesuaian kepada elektrod bercetak skrin serta pengesahan menggunakan sampel serum klinikal membuktikan kebolehgunaan platform ini untuk diagnostik POC. Secara keseluruhannya, kajian ini membuktikan bahawa FcγRIIA rekombinan boleh digunakan sebagai ligan bioafiniti baharu untuk pengimobilisasian antibodi secara terarah dalam biosensor elektrokimia. Pendekatan ini berjaya meningkatkan kepekaan, keboleh-ulangan dan prestasi dalam sampel kompleks, serta berpotensi besar untuk diaplikasikan dalam diagnostik POC, termasuk ujian hospital dan saringan penyakit berjangkit secara pantas.

CHARACTERISATION OF RECOMBINANT IGG BINDING PROTEIN, FCγRIIA FOR BIOSENSOR DEVELOPMENT

ABSTRACT

Antibodies play an important role in diagnostic applications due to their high specificity and affinity towards target antigens. However, in biosensor development, particularly for point-of-care (POC) diagnostics, the method of antibody immobilisation on sensor surfaces remains a critical bottleneck influencing assay sensitivity, reproducibility, and overall performance. Traditional techniques, such as passive adsorption or random covalent coupling, often result in poorly oriented and unstable antibodies. Consequently, assays showed high background noise, reduced binding activity, poor reproducibility, and relatively high detection limits, typically in the microgram-per-millilitre to high nanogram-per-millilitre range. Such shortcomings limit the reliability and clinical application of electrochemical immunosensors. To address these challenges, this study investigated the application of recombinant human IgG Fc receptors, namely FcγRIA, FcγRIIA, and the neonatal Fc receptor (FcRn), as site-specific antibody capture ligands for electrochemical biosensing. The receptors were expressed in a mammalian expression system to ensure proper folding and post-translational modifications. However, the recombinant protein secretion was limited by intracellular retention, likely due to the presence of unmodified endogenous signal peptides and hydrophobic transmembrane domains. Following purification, the receptors were immobilised onto gold electrodes *via* thiol-gold chemistry to facilitate directional IgG binding through the Fc region. The receptor-functionalised electrodes were incorporated into electrochemical sensor platforms for detecting two model antigens: interferon-gamma (IFN-γ) and *Salmonella* Typhi hemolysin E (HlyE).

Sensor performance was evaluated using square wave voltammetry (SWV) and cyclic voltammetry (CV), with analytical metrics including limit of detection (LOD), limit of quantification (LOQ), and coefficient of variation (COV) calculated to assess sensitivity and reproducibility. Among the tested receptors, FcγRIIA demonstrated superior performance as an antibody capture ligand, with minimal non-specific signal. The platform achieved LODs of 2.23 ng/mL for IFN-γ in buffer and 1.71 ng/mL for HlyE serum matrices, with COV values below 15 %. Its adaptation to screen-printed electrodes and validation with clinical serum samples further highlighted the applicability of this system for POC diagnostics. In summary, this study established a robust, site-specific antibody immobilisation strategy using recombinant FcγRIIA as functional bioaffinity ligands for electrochemical immunosensors. By enhancing sensitivity, reproducibility, and applicability in complex samples, this platform demonstrates strong potential for integration into POC diagnostics, with future applications in hospital-based testing and rapid infectious disease screening.

CHAPTER 1

INTRODUCTION

1.1 Research background

Fragment crystallisable gamma receptors (FcγRs) are the integral membrane glycoproteins predominantly expressed on immune cells surface. These receptors specifically recognise the Fc fragment of immunoglobulin G within immunocomplexes (ICs). The crosslinking of the receptors with IgG-ICs will trigger numerous signalling cascades, including phagocytosis, antibody-dependent cellular cytotoxicity (ADCC), and production of cytokine (Gan et al., 2024). FcγRs are classified based on their intracellular signalling motifs. For examples, FcγRIA (CD64), FcγRIIA (CD32A), FcγRIIC (CD32C), and FcγRIIIA (CD16A) are activating receptors, each equipped with an immunoreceptor tyrosine-based activation motif (ITAM). In contrast, FcγRIIB (CD32B) is the only inhibitory receptor in human FcγR family, having an immunoreceptor tyrosine-based inhibition motif (ITIM) in its cytoplasmic tail.

Structurally, FcγRs exhibit different affinities towards different subclasses of IgGs, categorised as high-affinity (FcγRIA) and low-affinity receptors (FcγRIIA, FcγRIIB, FcγRIIC, FcγRIIIA, and FcγRIIIB). Although all FcγRs interact with IgG-ICs with great avidity, only FcγRI strongly binds to monomeric IgG (dissociation constant, $K_d \approx 10^8\ M^{-1}$) (Hayes et al., 2016). Conversely, FcγRII and FcγRIII, with lower binding affinity ($K_d \approx 10^6\ M^{-1}$), more rely on the avidity effects for IgG interactions. The classical FcγRs binding mechanism is mainly mediated by their α-subunits, which are composed of two or three C2-type Ig-like domains (Nimmerjahn & Ravetch, 2011). These domains recognise the hinge-proximal C_H2 domain of Fc fragment, facilitating 1:1 binding with IgG Fc (Bournazos et al., 2016). Notably, the

presence of an extra Ig-like domain in FcγRI stabilizes its structure and projects itself towards extracellular area, making it more accessible for ligand binding (Lu et al., 2011).

In addition, the neonatal Fc receptor (FcRn) also binds Fc fragment of IgGs in a pH-dependent manner. FcRn is a heterodimer made up of a major histocompatibility complex class I (MHC-I)-like α-chains (p51) and a non-covalently linked β2-microglobulin light chain (p14) (Pyzik et al., 2019). In humans, FcRn plays a fundamental role in passive immunity, especially in the transport of maternal IgG across placenta into foetal circulation. Moreover, this receptor also regulates IgG homeostasis (Pyzik et al., 2015). In acidic endosomes (pH 5.5-6.0), FcRn binds to IgGs to protect IgG from intracellular degradation. IgG is then returned back into the bloodstream after reaching at physiological pH (pH 7.4). This recycling mechanism extends the serum IgG half-life (approximately 19-23 days), making FcRn a target for therapeutic antibody engineering (Pyzik et al., 2023).

Although the effector functions of FcγRs in immune response and its function as a therapeutic target have been thoroughly studied, their potential as an IgG affinity ligand has gained increasing attention (Boesch et al., 2018; Capkin et al., 2022; Çapkın et al., 2023; Kim et al., 2014; Ng et al., 2018). Previous studies had evaluated FcγRI as a binding ligand for monoclonal antibody capture. Kinetic analysis revealed that FcγRI exhibited a high affinity (K_d in the picomolar range) for human IgG1 under optimised conditions, suggesting its potential as a binding ligand for antibody purification and sensing applications (Capkin et al., 2022). Besides, in a study using surface plasmon resonance (SPR) analysis, FcγRI successfully captured the monoclonal antibody Humira (ADA) and detected the target antigen, tumour necrosis factor alpha (TNF-α) with a K_d of 0.63 ± 0.008 nM (Çapkın et al., 2023). Similarly,

Ng et al. (2018) also used human FcRn to immobilise antibodies in an oriented manner in immunoassays. The study demonstrated that FcRn has potential to be a novel antibody-binding protein for antibody immobilisation as it resulted in higher signal generation in a sandwich ELISA assay, compared to traditional physisorption approach (Ng et al., 2018). The increased efficiency in antibody binding and assay sensitivity highlighted the potential of these receptors in biosensing applications.

Electrochemical biosensor is an integral component of modern analytical chemistry, which utilised the interactions between bioreceptors and electrochemical transducer to quantitatively or semi-quantitatively analyse the target molecules. These biosensors are cheap, highly sensitive, and rapid in response, making them ideal for point-of-care (POC) diagnostics, specifically in label-free and miniaturised applications (Baranwal et al., 2022; Suhito et al., 2020).Typically, various electrode materials, including carbon, graphene, and gold, are well-studied. Gold, in particular, is widely used because it can form stable gold-thiol (Au-S) bonds, allowing easy surface fabrication with thiolated molecules (Zamani et al., 2022). Square wave voltammetry (SWV) is one of the most sophisticated voltametric techniques. It is not only highly sensitive, but also provides information regarding the electrode mechanisms and kinetic information on rapid electrode processes (Deffo et al., 2024; Megale & De Souza, 2023). Cyclic voltammetry (CV) is also widely applied in biosensors applications, particularly for investigating the redox processes and characterising the electrochemical properties of modified electrodes (Elgrishi et al., 2018).

Therefore, this study aims to express and characterise FcγRIA, FcγRIIa, and FcRn using a mammalian expression system. These receptors were evaluated as binding ligands to control the orientation of immobilised IgGs in the development of

a biosensor platform. The receptors were coated onto a fabricated gold electrode surface to immobilise IgG and facilitate the detection of target antigens, specifically Interferon gamma (IFN-γ) and hemolysin E (HlyE). The versatility and the performance of the sensor platform were assessed using SWV and CV, while the limit of detection (LOD), limit of quantification (LOQ), and coefficient of variation (COV) were used to evaluate the analytical reliability of the system. By advancing low-cost, reliable, and accessible diagnostic platforms, this study also contributed to SDG 3: Good Health and Well-being, through promoting equitable healthcare solutions, and SDG 9: Industry, Innovation, and Infrastructure, fostering innovation in biomedical technology.

1.2 Problem statement

The interaction between biomolecules and solid-support interfaces is crucial for biorecognition reactions, affecting the detection of analytes such as DNA, antibody, antigen, and cell (Al-Qaoud et al., 2024; Ma et al., 2023; Patolsky et al., 1999). These reactions induce substantial changes at three levels: the immobilised macromolecule, the macromolecule-fluid interface at the boundary layer, and the bulk fluid surrounding the macromolecule. Consequently, they influence the electrical and optical properties, such as impedance, current, and voltage.

Electrochemical immunosensors are one of the biosensor technologies that have drawn increasing attention due to their ease of use, portability, affordability and rapid detection (Mehrvar & Abdi, 2004; Ozer et al., 2020; Soares et al., 2022). Despite these advantages, electrochemical immunosensors still face limitations such as relatively high detection limits, poor reproducibility, and elevated background signals

(Kusnezow & Hoheisel, 2003; Park, 2019). These technical constraints are strongly influenced by the method of antibody immobilisation, particularly the orientation and stability of antibodies at the sensor interface (Gan et al., 2023; Gao et al., 2021).

Traditional antibody immobilisation through physisorption frequently results in non-specific binding, random orientation, and poor stability due to weak secondary bonds. As a result, the assay sensitivity is reduced and background signals are increased (Kusnezow & Hoheisel, 2003; Park, 2019; Sharafeldin et al., 2019). To circumvent the hurdle, various orientation-controlled strategies, such as surface modification, affinity-based techniques, and covalent attachment, have been developed. However, each method presents drawbacks in terms of cost, complexity, or antibody compatibility (Gan et al., 2023). While Fc binding proteins, such as Protein A and Protein G, have been employed to improve antibody orientation, they also showed non-specific interactions with other serum proteins and the IgG Fab part (Kamat et al., 2020; Sjöbring et al., 1989). This further increased the potential of false positive results in complex biological matrices.

In this context, FcγRs and FcRn represent promising alternatives. These receptors demonstrate high specificity and affinity towards the Fc part of IgG, thereby offering site-oriented immobilisation of antibodies. Importantly, FcγRI and FcRn display high-affinity binding to monomeric human IgG ($K_d \approx 10^8$ M^{-1}), while FcγRII and FcγRIII, although lower in affinity ($K_d \approx 10^6$ M^{-1}), achieve strong avidity for immune complexes (Hayes et al., 2016). Their strong binding affinity for IgGs from other species, like mouse and rabbits, was also reported in earlier studies (Bruhns et al., 2009; Szikora et al., 2017; Temming et al., 2020; Wang et al., 2022). Nonetheless, their integration into electrochemical sensor platforms and data on their effectiveness in enhancing sensitivity and reproducibility are limited. Therefore, this study was

aimed to develop an electrochemical immunosensor platform using recombinant FcγRIA, FcγRIIA, and FcRn as antibody binding ligands. By evaluating their structural and functional characteristics, this study seeks to enhance the performance and reliability of electrochemical immunosensor, overcoming the challenges of current antibody immobilisation strategies.

1.3 Research Objectives

1.3.1 Main Objective

To develop an electrochemical immunosensor using IgG Fc receptor as binding ligand for the detection of biomarkers.

1.3.2 Specific Research Objectives

1. To clone the genes encoding IgG Fc binding proteins (FcγRIA, FcγRIIA, and FcRn) into a mammalian stable expression vector (pcDNA5/FRT).
2. To express, purify and characterise the recombinant IgG Fc binding proteins structurally and functionally.
3. To modify the gold working electrode with the recombinant IgG Fc binding proteins for directional antibody immobilisation and determine the optimum conditions for antigen detection.
4. To evaluate the analytical performance of the fabricated electrochemical immunosensor in terms of limit of detection, reproducibility and cross reactivity.

CHAPTER 2

LITERATURE REVIEW

2.1 Structure and classification of immunoglobulins

Immunoglobulins (Igs), or better known as antibodies, are antigen-specific glycoproteins secreted by B lymphocytes in response to foreign antigens in circulation. They are important components of the adaptive immune system, assisting body to eliminate or neutralise invading pathogens and other foreign substances (Meffre et al., 2000). Antibodies are classified into five groups, namely IgA, IgD, IgE, IgG, and IgM. Each group has unique structural, functional characteristics, and distribution within the body (Woof & Burton, 2004).

An immunoglobulin monomer, which has a molecular weight of approximately 150 kDa, consists of four polypeptide chains: two identical heavy (H) chains and two identical light (L) chains (Ma & O'Kennedy, 2015) (**Figure 2.1**). These chains are linked by intermolecular disulfide bonds, which stabilise the structure of antibodies. Each polypeptide chain is consisting of two important regions: the variable (V) region and the constant (C) region. The V region is responsible for antigen recognition, whereas the C region defines the effector function of the antibody. The V region, located at the N-terminal of both H and L chains, varies from antibody to antibody. Within this region, the complementarity-determining regions (CDRs) form the antigen binding sites, allowing antibody to recognise specific epitopes with high affinity and specificity (Brezski & Georgiou, 2016; Narciso et al., 2011). Conversely, the C region at the C-terminal, is highly conserved within each isotype and controls antibody interactions with Fc receptors (FcRs), complement proteins and other immune effectors (Janda et al., 2016).

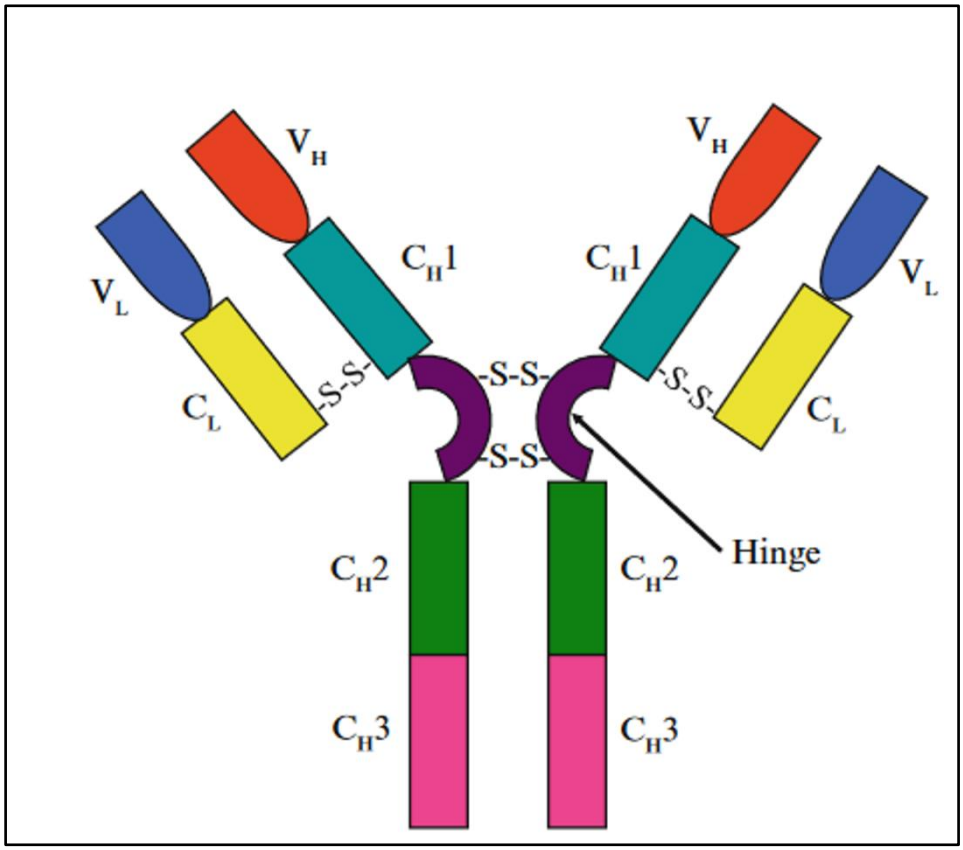

Figure 2.1 The structure of a basic immunoglobulin monomer (Ma & O'Kennedy, 2015).

Structurally, each L chain has one variable domain (V_L) and one constant domain (C_L), whereas each H chain contains one variable region (V_H) and three or four constant domains (C_H1-C_H3 or C_H1-C_H4), depending on isotypes. Notably, IgM and IgE consist of an additional domain (C_H4), altering their effector functions. Functionally, an antibody is classified into two major parts: the antigen-binding (Fab) fragment and the fragment crystallisable (Fc) region. The Fab region contains the V_H, V_L, C_H1, and C_L domains, which mediate the antigen binding and detection. In contrast, the remaining C_H domains (C_H2-C_H3 or C_H2-C_H4) make up the Fc region. This region is responsible for the interaction with Fc receptors to trigger various immune response (Brezski & Georgiou, 2016). Modifications in the Fc fragment can substantially influence the

antibody function, including antigen-binding efficiency, stability, and immune activation (Torres & Casadevall, 2008).

While all Ig isotypes share a fundamental structure, they demonstrate different functional specialisations (Schroeder & Cavacini, 2010). For example, IgG is the most abundant antibody in serum, and it plays an important role in long-term immunity, opsonisation and Fc receptor-mediated effector functions. IgM is the first antibody produced during an immune response, and it participate in immunoregulation. Moreover, IgA is mainly found in mucosal secretions to direct neutralise or prevent the binding of virus or toxins to the mucosal surface. IgE, which has the lowest serum concentration, is involved in hypersensitivity and allergic reactions. Next, IgD, although less well understood, is believed to be involved in antigen-triggered lymphocyte differentiation (Vladutiu, 2000).

The functional diversity of immunoglobulins is influenced by their structural organisation. While IgG, IgE, and IgD typically exists as monomers, IgA is commonly found as a dimer, and IgM predominantly exists as a pentamer (Schroeder & Cavacini, 2010). These polymeric forms enhance the antigen-binding avidity and stability under physiological conditions.

2.1.1 Immunoglobulin G (IgG)

The most abundant antibody in human serum is immunoglobulin G (IgG), which makes up over half of the total immunoglobulins (Taylor et al., 2022). IgG is secreted during the secondary immune response to antigens, and it exhibits remarkably specificity and efficiency in immunological functions. This monomeric glycoprotein, with a molecular weight of around 150 kDa, plays a critical role in immunity by neutralising invading pathogens, facilitating opsonisation, and triggering effector

responses through interactions with immune cells. A distinctive feature of IgG is its ability to cross the placental barrier, making it the only antibody isotype capable of providing passive immunity to the foetus and newborn (Borghesi et al., 2014). Due to the high specificity and stability of IgG, it is widely employed in clinical diagnostics, therapeutic applications, and biomedical research (Jiang et al., 2020; Jolles et al., 2014; Lee et al., 2019; Shepard et al., 2017).

Human IgG is further grouped into four subclasses: IgG1, IgG2, IgG3, and IgG4, based on the different antigenic determinants found in their heavy chain constant regions and related physiological roles (**Figure 2.2**). The subclasses share more than 90% amino acid sequence homology. However, the most notable variation is the amount of cysteine residues likely engaged in the intramolecular disulfide bonds (IgG1: 2; IgG2: 4; IgG3: 11; IgG4: 2) (Hamilton, 2014). The subclasses also vary in the number of amino acids in the hinge region (IgG1: 15; IgG2: 12; IgG3: 62; IgG4: 21). As a result, the functional characteristics vary significantly among four subclasses, especially in antigen affinity, complement activation, Fc receptor binding, and immune complex formation (Engelhart et al., 2017; Vidarsson et al., 2014). IgG1 and IgG3 are the most effective in activating complement and interacting with Fc gamma receptors (FcγRs), playing key roles in immune responses against antigens. On the contrary, IgG2 is largely involved in responses against carbohydrate antigen (Bournazos & Ravetch, 2015), whereas IgG4 responsible to regulate immune tolerance (Rispens & Huijbers, 2023).

The interaction of IgG with Fcγ receptors (FcγRs) on immune cells is essential in modulating immune responses. The Fc fragment of IgG, composed of the C_H2 and C_H3 domains, showed structural flexibility that influences its interactions with FcγRs. The C_H3 domains which form homodimers at the C-terminal region and the disulfide bonds in the C_H2-proximal hinge region contribute to the horseshoe-like conformation

of the Fc region (Bournazos et al., 2016). An important feature of the Fc region is the N-linked glycan at Asn-297 in the C_H2 domain (Schroeder & Cavacini, 2010; Torres & Casadevall, 2008). This Fc-associated glycan is essential for maintaining a conformation that allows FcγR binding. Alterations in the glycan structure, such as the addition of fucose, galactose, N-acetylglucosamine, or sialic acid residues, alter the affinity of the Fc domain for different classes of FcγRs (Daëron, 2014). As a result, the immune functions of IgG altered, influencing antibody-dependent cellular cytotoxicity (ADCC), phagocytosis, and immune regulation.

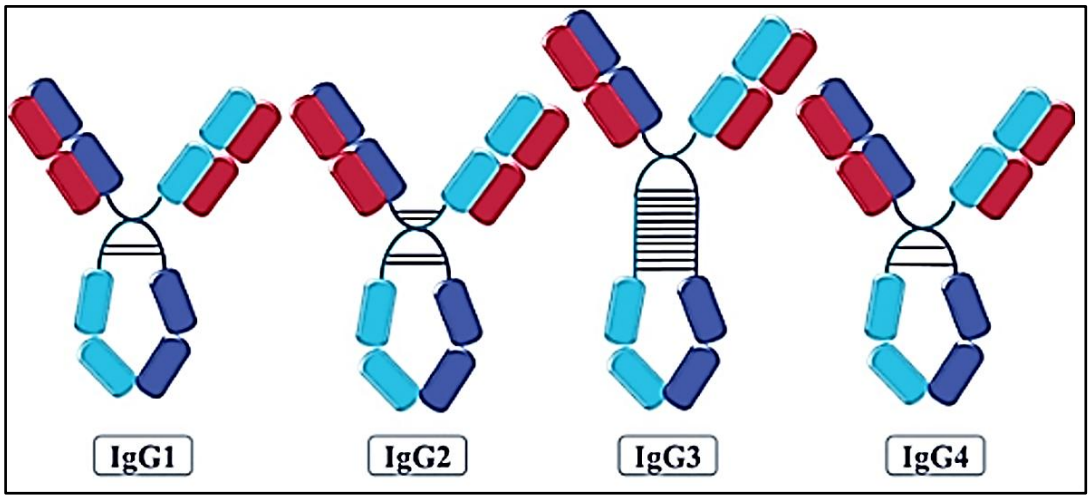

Figure 2.2 Subclasses of human IgG: IgG1, IgG2, IgG3, and IgG4 (Napodano et al., 2021).

2.2 Fc receptors (FcRs)

2.2.1 Fc gamma receptors (FcγRs)

Fc gamma receptors (FcγRs) are a class of immunoreceptors that specifically recognise the Fc region of human IgG (hIgG) molecules. These receptors are type I transmembrane glycoproteins, which have N-terminal in extracellular space and C-terminal in cytoplasmic region. Human FcγRs are classified into three groups: FcγRI

(CD64), FcγRII (CD32) and FcγRIII (CD16). Among them, FcγRI, is the only receptor that can bind to monomeric human IgG1, IgG3, and IgG4 with high affinity (Bruhns et al., 2009). Nevertheless, all FcγRs interact with hIgG molecules in the form of immunocomplexes (ICs), leading to activation of downstream signalling cascades. These signalling pathways initiate immune functions, such as phagocytosis, cytokines production, degranulation, and ADCC, to effectively eliminate pathogens and regulate immune responses (Garcia-Garcia & Rosales, 2009; Nimmerjahn & Ravetch, 2011).

The FCGR gene family on chromosome 1 encodes the human FcγRs (Geraghty et al., 2019). FcγRIA is encoded by FCGR1A gene, which is found at chromosome 1q21, whereas the genes for the remaining FcγRs (FCGR2A, FCGR2B, FCGR2C, FCGR3A and FCGR3B) are clustered at the 1q23 loci (Nagelkerke et al., 2019). These receptors demonstrate structural differences, varying expression patterns, and different affinities towards IgG subclasses.

Structurally, FcγRs contain an α-subunit responsible for IgG ligand binding. This subunit consists of two or three C2-type Ig-like domains (**Figure 2.3**), which target the C_H2 domain in Fc region of IgG and mediate 1:1 binding interaction with IgG (Bournazos et al., 2016). Additionally, an accessory chain protein, known as Fc receptor gamma chain (FcR γ chain) is linked to the α-subunit of FcγRIA and FcγRIIIA. This chain contains an activating signalling motif and participates in Fcε receptor signalling (Brandsma et al., 2016). For the FcγRII family, they function independently of the FcR γ chain. Instead, they act as single-chain receptors with activation and inhibition signalling capabilities. Moreover, FcγRIIIB is a glycophosphatidylinositol (GPI) anchored receptor that lacks a cystoplasmic domain (Rosales & Uribe-Querol, 2013). Although it shares 97% amino acid sequence homology with FcγRIIIA, it is unable to transduce intracellular signals. Thus, its signalling function often relies on accessory

chains (ζ chain or FcR γ chain) or other immune receptors (FcγRIIA) (Bournazos et al., 2016).

FcγRs are extensively expressed on various immune cells, including monocytes, macrophages, neutrophils, and dendritic cells (**Table 2.1**). Monocytes and macrophages are expressing all activating FcγRs, whereas neutrophils express FcγRIIIB (Alemán et al., 2021). In humans, natural killer (NK) cells mainly produce FcγRIIIA (Bruhns, 2012) and FcγRIIC in some populations (Gorlova et al., 2018; Lassaunière & Tiemessen, 2016; van der Heijden et al., 2012). Dendritic cells (DCs) also express most of the FcγRs (Ravetch & Bolland, 2001). In contrast, B lymphocytes express inhibitory FcγRIIB, Interestingly, recent findings suggested that FcγRIIB is upregulated on memory CD8 T cells under chronic inflammation, such as chronic viral infections and cancer. This upregulation is associated with the elevated level of serum Fgl2 protein (Morris et al., 2020), indicating a potential role of FcγRIIB in the immune system.

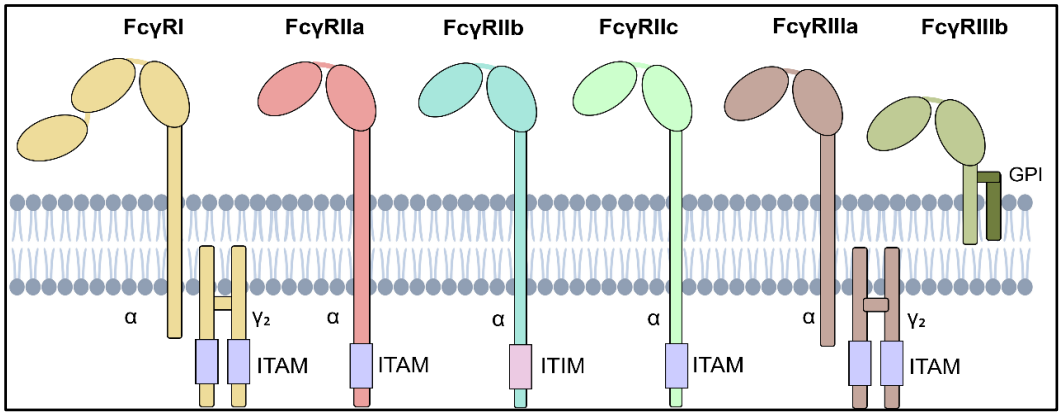

Figure 2.3 Structure of human FcγRs (Gan et al., 2024).

Table 2.1 Characteristics and expression of human FcγRs and FcRn (Bruhns, 2012; Hamdan et al., 2020; Nagelkerke et al., 2019; Nimmerjahn & Ravetch, 2011).

FcRs	Gene location	Structure	Variant	Cell distribution
FcγRIA (CD64)	1q21	αγγ	-	Mast cells, macrophages, monocytes, neutrophils, eosinophils, dendritic cells
FcγRIIA (CD32A)	1q23	α	H_{131} R_{131}	Mast cells, basophils, macrophages, monocytes, neutrophils, eosinophils, platelets, dendritic cells
FcγRIIB (CD32B)	1q23	α	I_{232} T_{232}	Basophils, macrophages, monocytes, neutrophils, dendritic cells
FcγRIIC (CD32C)	1q23	α	-	NK cells *
FcγRIIIIA (CD16A)	1q23	αγγ	V_{158} F_{158}	Macrophages, monocytes, NK cells
FcγRIIIB (CD16B)	1q23	α-GPI	NA1 NA2 SH	Neutrophils, basophils
FcRn	19q13	α-β2m	-	Macrophages, monocytes, dendritic cells, neutrophils, endothelium, syncytiotrophoblasts

*Expression only found in approximately 20% of the population.

2.2.2 Signalling pathway

The majority of FcγRs in humans are activating receptors. These receptors contain an immunoreceptor tyrosine-based activation motif (ITAM), which is essential for initiation of downstream signalling and receptor assembly (Rosales, 2017). Activating FcγRs undergo aggregation when bind to IgGs, subsequently activating and engaging Src family kinases (SFKs), such as Lyn and Fyn (Mkaddem et al., 2017). By phosphorylating the ITAM motif, these kinases then activate Syk kinase. After that,

various proteins, such as Bruton's tyrosine kinases (Btk), phospholipase Cγ1 (PLCγ1), and phosphatidylinositol 3-kinases (PI3K), are then activated to control various immune functions like phagocytosis, oxidative burst, ADCC and antigen presentation (Ben Mkaddem et al., 2019; Garcia-Garcia & Rosales, 2009) (**Figure 2.4**). The molecular mechanism of these cellular activations leads to elimination of pathogen and immune homeostasis.

On the other hand, FcγRIIB contains an immunoreceptor tyrosine-based inhibition motif (ITIM) in its cytoplasmic tail that suppresses the effector cells activity (Daëron & Lesourne, 2006; Getahun & Cambier, 2015). Upon co-aggregation with activating receptors, the phosphorylated-ITIM domain recruits tyrosine phosphatases and inositol phosphatases to adversely regulate several cellular activities (**Figure 2.4**). Through these processes, FcγRIIB is particularly important in preventing autoimmune disorders by inhibiting B-cell receptor signalling (Takai, 2002).

Furthermore, FcγRIIIB does not have an intracellular signalling motif and does not directly activate signalling cascades. But it contributes to immune responses through its ability to bind to IgG-opsonised targets in neutrophils (Chen et al., 2012). It acts as a decoy receptor to compete with FcγRIIA, regulating neutrophil-mediated ADCC (Treffers et al., 2018). Studies also suggested that FcγRIIIB promotes granule proteins exocytosis and calcium influx in neutrophils (Huizinga et al., 1990; Vossebeld et al., 1995).

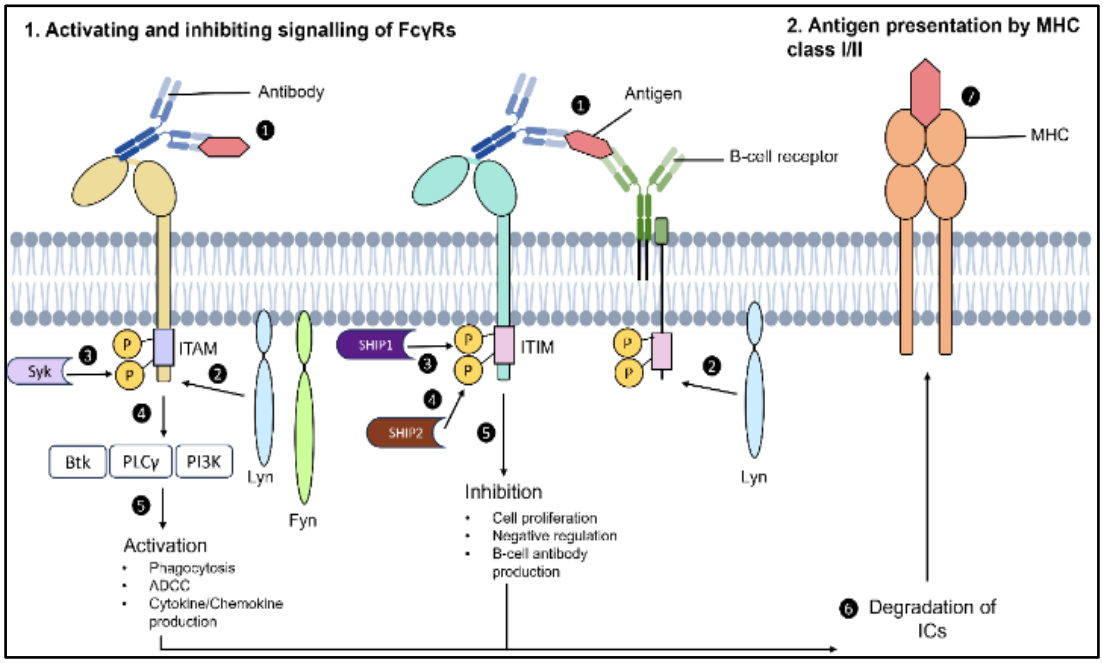

Figure 2.4 The functions and signalling pathway of activating and inhibitory FcγRs (Gan et al., 2024).

2.2.3 Neonatal Fc receptor (FcRn)

The neonatal Fc receptor (FcRn) was first hypothesized by Rogers Brambell as a part of Fc receptor system responsible for transplacental IgG transport, based on studies of maternal antibody transfer. FcRn is a non-classical Fc gamma receptor, which plays a role in transporting maternal IgG to the foetus, controlling IgG and albumin homeostasis (Roopenian & Akilesh, 2007), and facilitating antigen presentation through the formation of IgG-ICs (Baker et al., 2014). It is broadly expressed on numerous immune and non-immune cells, such as macrophages, monocytes, dendritic cells, neutrophils, endothelium, and syncytiotrophoblasts (Bruhns, 2012; Corrales-Aguilar et al., 2014) (**Table 2.1**). In human, FcRn is encoded by the FCGRT gene on chromosome 19q13 (Pyzik et al., 2015).

FcRn has a heterodimer structure composed of a transmembrane-anchored α-chain and a non-covalently linked β2-microglobulin (β2m) light chain. The α-heavy chain (40 kDa) has three extracellular domains (α1, α2, α3). Similar to the structure of

MHC-I, the α1 and α2 domains form a structural platform with eight-stranded antiparallel β-sheets and two α-helices (West & Bjorkman, 2000) (**Figure 2.5**). However, it cannot present antigenic peptides to T cells due to its closed and non-functional peptide-binding groove (Pyzik et al., 2019). The β2m light chain interacts with the heavy chain to stabilize the structural platform and ensure the proper folding of FcRn. According to Pyzik et al. (2015), this structure enables FcRn to bind IgG and albumin in a pH-dependent manner, ensuring their intracellular recycling and extending their serum half-life (approximately 19-21 days in humans).

The key amino acids on the IgG Fc region, such as Ile-253, Thr-254, His-310, His-433, and His-435, mediate the FcRn-IgG interaction. These residues form salt bridges and hydrogen bonds with Glu-115 and Asp-130 residues on FcRn (Martin et al., 2001; Oganesyan et al., 2014). In acidic endosomes (pH 5.0-6.5), the histidine residues of IgG become protonated and thus enable electrostatic interaction with negatively charged residues (Glu-115 and Asp-130) of FcRn. At blood circulation with neutral pH (pH 7.2-7.4), deprotonation of histidine residues destabilises these interactions, resulting in dissociation of IgG from FcRn (**Figure 2.6**). This mechanism allows selective recycling of IgG while directing unbound proteins towards degradation (Pyzik et al., 2015). Structural studies revealed that FcRn binds IgG with a 2:1 stoichiometry, where two FcRn molecules interact with single IgG at independent sites (Abdiche et al., 2015). However, , 1:1 FcRn-IgG complexes can also form under non-equilibrium condition, suggesting that binding dynamics may vary physiologically (Popov et al., 1996; Sánchez et al., 1999).

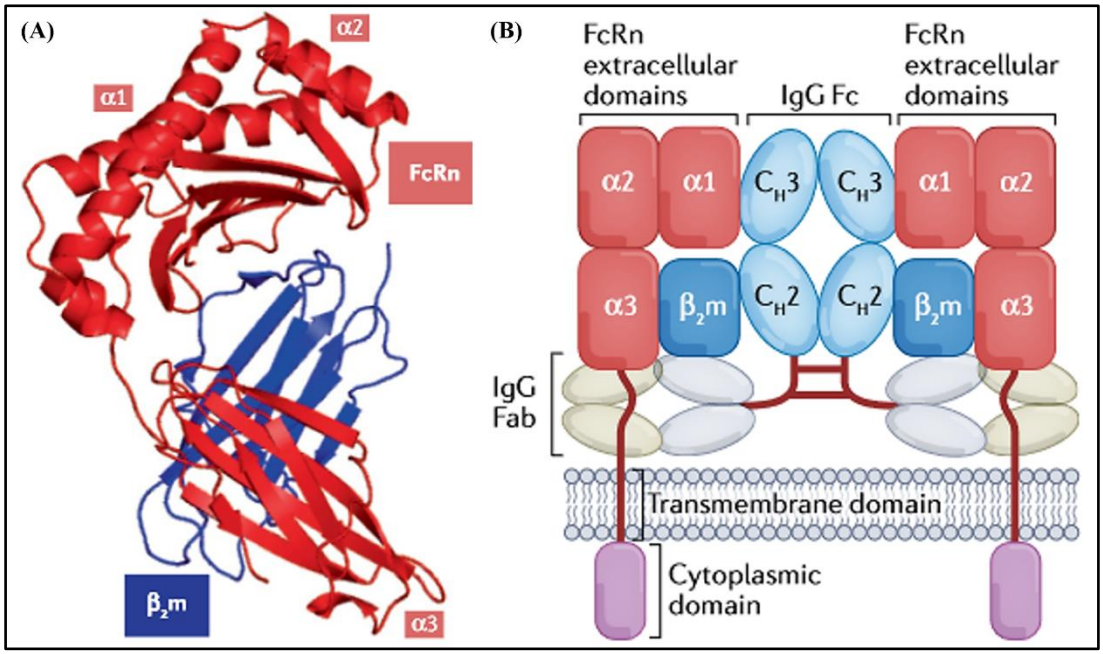

Figure 2.5 (A) Ribbon diagram of the human neonatal Fc receptor (FcRn) heavy chain (red) with indicated α1, α2, and α3 domains; and β2-microglobulin (blue) (PDB ID: 1EXU). (B) Cartoon representation of two FcRn–β2m heterodimers engaging monomeric IgG (in a T conformation) (Pyzik et al., 2023).

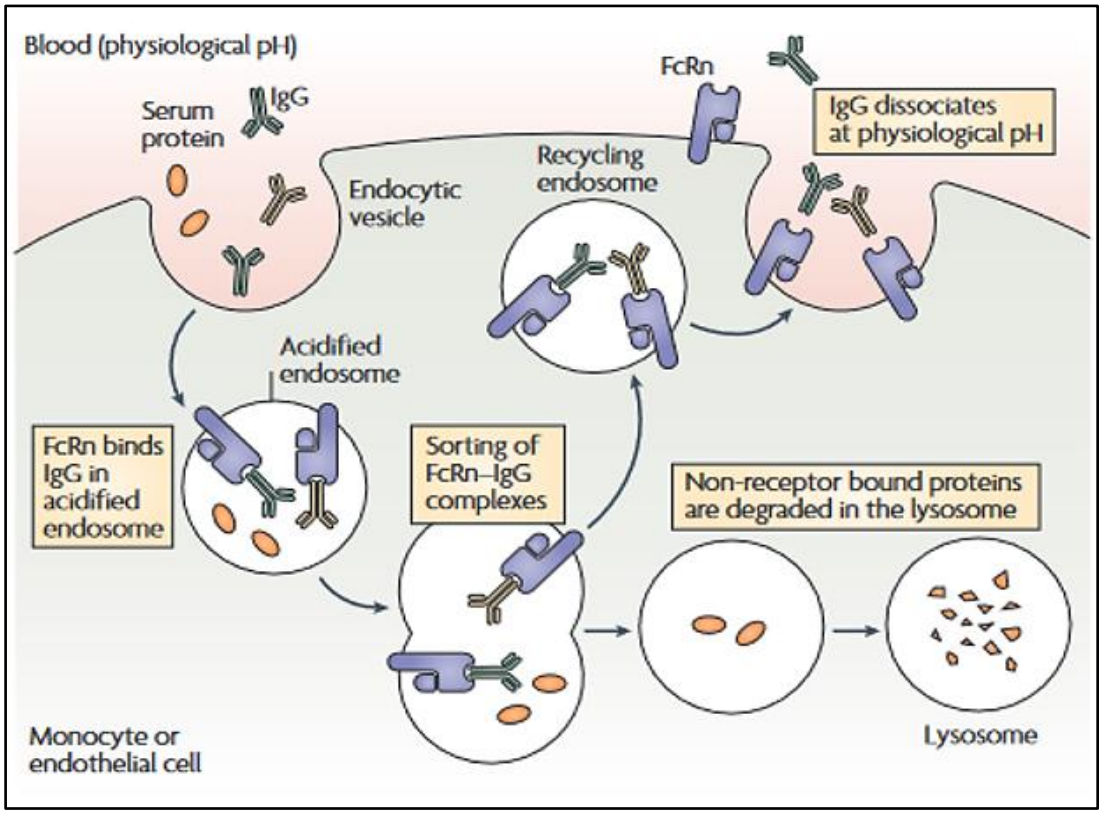

Figure 2.6 The FcRn receptor in endothelial cells and monocytes recycles IgG, extending its serum half-life by preventing lysosomal degradation (Roopenian & Akilesh, 2007).

In humans, FcRn transports maternal IgG across the placenta before birth, providing passive immunity to newborns. In rodents, FcRn is expressed in the neonatal intestinal epithelium to facilitate the absorption of IgG from ingested milk into the bloodstream. After birth, FcRn remains active in epithelial tissues in children and adults, assisting IgG transport across mucosal barriers, which is crucial for immune defence and targeted drug delivery. In contrast to typical FcγRs, FcRn mainly promotes antigen presentation by processing IgG-ICs (Baker et al., 2014). Endocytosis is initiated when the IgG-ICs attach to FcγRs on the surface of DCs. Within acidic endolysosomal compartments, FcRn replaces FcγRs and guides IgG-ICs into antigen-processing pathways. These complexes are processed and loaded onto MHC class I and II molecules, which are then displayed on the cell surface to activate T cells (Pyzik et al., 2023).

2.2.4 Binding affinity with IgGs

The interaction of IgG with Fc receptors (FcγRs and FcRn) is crucial for immune regulation because it influences immunological functions, such as IgG recycling, ADCC, and phagocytosis. These interactions rely on numerous parameters, such as receptor type, as well as subclasses, structural forms (monomeric or ICs), glycosylation patterns, and species origin of IgG (Bruhns et al., 2009; Ober et al., 2001; Wang et al., 2022). The binding of IgG to FcγRs and FcRn is mediated by certain amino acid residues in both the Fc region of IgG and the extracellular domains of receptors.

In human IgG (hIgG), several crucial residues in the Fc region, such as Pro-329 and Asn-297, contribute to FcγR binding. On the other hand, His-310 and His-435 residues are important for interaction with FcRn (Lu et al., 2011; Oganesyan et al., 2014). On the receptor side, Trp-104 and Trp-127 of FcγRI are important for IgG

binding as they form a sandwich-like structure around Pro-329 residue in the Fc region (Lu et al., 2011). In FcγRIIa, Trp-90 and Trp-113 trapped the Pro-329 residue, while Ser-129 and Lys-128 residues interact with the Asn-297 glycan (Ramsland et al., 2011). Moreover, the Glu-115, Glu-116, and Asp-130 residues in FcRn interact with His-310 and His-435 residues of IgG pH-dependently (Andersen et al., 2006; West & Bjorkman, 2000).

The ability of FcγRs to bind monomeric IgG is primarily limited to high-affinity receptor. Among human FcγRs, FcγRI exhibits the strongest affinity for monomeric hIgG, particularly hIgG1, hIgG3, and hIgG4, but not hIgG2 (Bruhns et al., 2009). In contrast, the low-affinity receptors, FcγRII and FcγRIII, require hIgG in ICs formation for stable interactions.

Furthermore, cross-species differences also influence the binding affinity of Fc receptors. For instance, human FcγRI preferentially binds to monomeric mouse IgG2a, IgG2c and IgG3, with weaker interactions observed in mouse IgG2b (Temming et al., 2020). Besides, FcγRII interacts with monomeric mouse IgG1, IgG2a and IgG2c, whereas FcγRIIIa (V158) only binds monomeric mouse IgG2a and IgG2c. Human FcRn shows strong binding to monomeric hIgG2, hIgG3, and hIgG4, meanwhile displaying moderate interaction with hIgG1 at acidic pH (pH 5.0-6.0) (Szikora et al., 2017). It also exhibits moderate affinity for monomeric rabbit IgG.

Unlike monomeric IgG, IgG-ICs, which form *via* multivalent antigen-IgG interactions, demonstrated higher binding affinity to low-affinity FcγRs (Wang et al., 2022). Generally, hIgG1, hIgG3, and hIgG4 ICs bind strongly to all human FcγRs and FcRn, following the rank order: IgG3 > IgG1 > IgG4 (**Table 2.2**). hIgG2 ICs exhibit more selective binding, mainly engaging FcγRIIa, FcγRIIb, FcγRIIIa (V158), and FcRn (Szikora et al., 2017; Wang et al., 2022).

Among mouse IgGs, IgG1 and IgG2b ICs primarily bind FcγRIIA, while FcγRIA and FcγRIIB are poorly bound. Mouse IgG2a and IgG2c ICs interact broadly with all FcγRs, except FcγRIIIB variants, however IgG3 ICs exhibit limited binding to FcγRIA and FcγRIIIA (V158). Besides, FcRn binds weakly to mouse IgG2a and IgG2b, showing limited interaction with mouse IgG (Ober et al., 2001).

On the other hand, all human FcγRs and FcRn are strongly bind to rabbit IgG ICs (Wang et al., 2022). Rat IgG1 and IgG2a ICs preferentially bind FcγRIIA and FcRn, whereas IgG2b ICs exhibit varied affinities among FcγRs. The binding of goat and guinea pig IgG ICs are weaker, where goat IgG IC mostly binds FcγRIIA, and guinea pig IgG IC favours FcγRIIIa. These variations in human FcγRs and FcRn cross-species binding have substantial implications for the development of novel therapeutics and diagnostics.

Table 2.2 Comparative overview of binding affinities of human FcγRs and FcRn for IgG-immunocomplexes (ICs) across different species (Bruhns et al., 2009; Bruhns, 2012; Neuber et al., 2014; Ober et al., 2001; Szikora et al., 2017; Temming et al., 2020; Wang et al., 2022).

Human FcγRs	Human				Mouse					Rabbit	Rat			Guinea pig	Goat
	IgG1	IgG2	IgG3	IgG4	IgG1	IgG2a	IgG2b	IgG2c	IgG3	IgG	IgG1	IgG2a	IgG2b	IgG	IgG
FcγRIA	+++	+	+++	++	+	+++	+	++	+	+	-	-	++	++	-
FcγRIIA (H131)	+++	++	+++	++	++	++	++	+++	-	++	+	+	+++	-	-
FcγRIIA (R131)	+++	+	+++	+	+++	++	++	+++	-	++	++	+	++	-	++
FcγRIIB	++	+	++	+	+	++	+	++	-	+	+	-	-	-	+
FcγRIIC	+	-	++	+	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
FcγRIIIA (F158)	++	-	+++	+	+	-	-	+	-	+++	-	-	+	+++	-
FcγRIIIA (V158)	+++	+	+++	++	+	++	-	+++	+	+++	-	-	+++	+++	+
FcγRIIIB (NA1)	+++	-	+++	+	-	-	-	-	-	+++	-	-	++	+++	-
FcγRIIIB (NA2)	+++	-	+++	+	-	-	-	-	-	+++	-	-	+	+++	-
FcγRIIIB (SH)	+++	-	+++	+	-	-	-	-	-	+++	-	-	+	+++	-
FcRn	+++	+++	++	+++	-	+	+	n/a	n/a	+++	+	+	-	+++	n/a

The binding affinities of FcγRs and FcRn for IgG-ICs were classified as no binding (-), weak (+), good (++), strong binding (+++), and not available (n/a).

2.3 Mammalian protein expression

A gene is the basic unit of genetic information essential to control the synthesis of polypeptides. Gene expression is the biological process by which genetic information is transcribed and translated to produce functional proteins (Khan, 2013). A suitable host system is required for the successful expression of a gene, as different hosts demonstrate varying efficiencies in post-translational modifications (PTMs), protein output, and scalability. To date, several expression systems have been developed for large-scale recombinant protein production, including *Escherichia coli* (*E. coli*), yeast, baculovirus-infected insect cells, and mammalian cells. Each system provides unique advantages in terms of cost-effectiveness, ease of use, and PTMs compatibility. The selection of an effective system depends on several factors, such as laboratory resources, nature of the protein, required yield, purification methods, and overall cost of production (Khan, 2013; Zhu, 2012).

Mammalian expression systems have broad application for the production of recombinant protein due to their ability to perform complex PTMs, such as glycosylation, phosphorylation, and proper protein folding, which are important for therapeutic and diagnostic applications (Baldi et al., 2007; Chapple et al., 2006). Unlike yeast and bacterial expression systems, mammalian expression systems can express protein with native-like structure to make sure the functional activity of proteins and reduce the risk of immunogenicity in clinical settings. The efficiency of a mammalian expression system is influenced by expression vectors, type of host cell line, culture conditions, and transfection techniques.

Chinese Hamster Ovary (CHO) cells are the most commonly employed mammalian expression system in industrial protein production due to their high

adaptability, rapid proliferation, and ability to achieve high cell density in serum-free media (Malm et al., 2022). They also have a lower susceptibility to human viral infections, thereby minimising the possibility of contamination in biopharmaceutical manufacturing. Besides, various gene amplification systems have been developed for CHO cells, enabling high-yield protein production and enhanced productivity (Lalonde & Durocher, 2017). However, a key shortcoming of CHO cells is the integration of non-human glycan structures, such as N-glycolylneuraminic acid (Neu5Gc) and terminal galactosyl-α1-3-galactosyl (α1-3-Gal) disaccharide, into glycoproteins (Diaz et al., 2009; Hatfield et al., 2023). These non-human glycosylation patterns can alter the biological activity of the proteins and induce hypersensitivity reactions. Variations in N-glycosylation of FcγRs expressed in CHO cells have been shown to reduce IgG binding affinity and increase antibody dissociation rates (Hayes et al., 2017; Zeck et al., 2011). For instance, CHO-derived FcγRIA and FcγRIIIA contained large and highly sialylated glycans, which have been reported to adversely affect the binding with IgG1 monoclonal antibody (Hayes et al., 2017).

To overcome the limitations associated with non-human glycosylation, Human Embryonic Kidney 293 (HEK293) cells have been established as an alternate host for recombinant protein production, particularly for proteins requiring human-specific PTMs. The HEK293 cell line was originally derived from the kidney of an aborted female embryo and was immortalised through the integration of a 4 kb adenoviral type 5 (Ad5) genome fragment, including the E1A and E1B genes, into chromosome 19 (Graham et al., 1977). These genetic modifications allow continuous cell proliferation by inhibiting apoptosis, modulating transcription and regulating cell cycle progression. To enhance the protein production efficiency, a number of HEK293-derived cell lines have been developed, including HEK293T, HEK293E, HEK293F and HEK293FTM